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Chronic lead exposure alters presynaptic calcium regulation and synaptic facilitation in *Drosophila* larvae

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Abstract

Prolonged exposure to inorganic lead (Pb^{2+}) during development has been shown to influence activity-dependent synaptic plasticity in the mammalian brain, possibly by altering the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). To explore this possibility, we studied the effect of Pb^{2+} exposure on $[Ca^{2+}]_i$ regulation and synaptic facilitation at the neuromuscular junction of larval *Drosophila*. Wild-type *Drosophila* (CS) were raised from egg stages through the third larval instar in media containing either 0, 100 μM or 250 μM Pb^{2+} and identified motor terminals were examined in late third-instar larvae. To compare resting $[Ca^{2+}]_i$ and the changes in $[Ca^{2+}]_i$ produced by impulse activity, the motor terminals were loaded with a Ca^{2+} indicator, either Oregon Green 488 BAPTA-1 (OGB-1) or fura-2 conjugated to a dextran. We found that rearing in Pb^{2+} did not significantly change the resting $[Ca^{2+}]_i$ nor the Ca^{2+} transient produced in synaptic boutons by single action potentials (APs); however, the Ca^{2+} transients produced by 10 and 20 Hz AP trains were larger in Pb^{2+} -exposed boutons and decayed more slowly. For larvae raised in 250 μM Pb^{2+} , the increase in $[Ca^{2+}]_i$ during an AP train (20 Hz) was 29% greater than in control larvae and the $[Ca^{2+}]_i$ decay τ was 69% greater. These differences appear to result from reduced activity of the plasma membrane Ca^{2+} ATPase (PMCA), which extrudes Ca^{2+} from these synaptic terminals. These findings are consistent with studies in mammals showing a Pb^{2+} -dependent reduction in PMCA activity. We also observed a Pb^{2+} -dependent enhancement of synaptic facilitation at these larval neuromuscular synapses. Facilitation of EPSP amplitude during AP trains (20 Hz) was 55% greater in Pb^{2+} -reared larvae than in controls. These results showed that Pb^{2+} exposure produced changes in the regulation of $[Ca^{2+}]_i$ during impulse activity, which could affect various aspects of nervous system development. At the mature synapse, this altered $[Ca^{2+}]_i$ regulation produced changes in synaptic facilitation that are likely to influence the function of neural networks.

Keywords

Pb^{2+} ; synapse; *Drosophila*; larvae; calcium; facilitation

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Introduction

Chronic Pb^{2+} exposure can produce changes in the structure and function of the mammalian brain (Costa et al., 2004; Toscano and Guilarte, 2005). This is particularly true for synapses where Pb^{2+} exposure during prenatal and/or postnatal development altered paired-pulse facilitation and long-term potentiation in the rat hippocampus (Altmann et al., 1993; Gilbert et al., 1996; Gilbert and Mack, 1998; Lasley et al., 1993; Ruan et al., 1998). In addition, Pb^{2+} exposure during postnatal development (10 mg/ml Pb acetate in fluids or 4% Pb carbonate in food) has been shown to alter the density of dendritic spines and presumably synapses in the brains of rats and cats (Kiraly and Jones, 1982; Petit and LeBoutillier, 1979). The effects of Pb^{2+} exposure on synaptic development and plasticity could result from alterations in $[\text{Ca}^{2+}]_i$ regulation. Intracellular Ca^{2+} can influence multiple steps in synaptic development; e.g., growth cone guidance (Jin et al., 2005), synapse formation (Xu et al., 2009) and synapse elimination and stabilization (Lohmann and Bonhoeffer, 2008; Pratt et al., 2003). At the mature synapse, altered $[\text{Ca}^{2+}]_i$ regulation can influence long and short-term forms of synaptic plasticity (MacDonald et al., 2006; Zucker and Regehr, 2002).

The effects of Pb^{2+} on Ca^{2+} influx and efflux are well documented. Acute exposure to Pb^{2+} (nanomolar to micromolar) blocks Ca^{2+} influx through both invertebrate (Audesirk and Audesirk, 1989; Busselberg et al., 1990) and mammalian voltage-dependent Ca^{2+} channels (Audesirk and Audesirk, 1991; Audesirk and Audesirk, 1993; Busselberg et al., 1993; Evans et al., 1991; Peng et al., 2002). Also, micromolar concentrations of Pb^{2+} can inhibit the extrusion of Ca^{2+} by the plasma membrane Ca^{2+} ATPase (PMCA) in humans and rats (Bettaiya et al., 1996; Mas-Oliva, 1989; Sandhir and Gill, 1994) although the PMCA is stimulated by lower (nanomolar) Pb^{2+} concentrations (Ferguson et al., 2000; Mas-Oliva, 1989). However, it is not known whether the effects of Pb^{2+} exposure on synaptic development and plasticity are due to altered regulation of $[\text{Ca}^{2+}]_i$. In fact, the effect of Pb^{2+} exposure on the regulation of $[\text{Ca}^{2+}]_i$ has not been well characterized. For example, there are no studies examining the effect of Pb^{2+} exposure on the changes in $[\text{Ca}^{2+}]_i$ produced by impulse activity. It is difficult to examine the acute effects of Pb^{2+} on $[\text{Ca}^{2+}]_i$ regulation since Pb^{2+} can enter through voltage-dependent Ca^{2+} channels (Simons and Pocock, 1987; Tomsig and Suszkiw, 1991) and Ca^{2+} indicators can respond to both Ca^{2+} and Pb^{2+} (Kerper and Hinkle, 1997; Tomsig and Suszkiw, 1990). However, the long-term effects of chronic Pb^{2+} exposure on $[\text{Ca}^{2+}]_i$ can be measured in Pb^{2+} -free saline. In fact, fura-2 has been used to measure resting $[\text{Ca}^{2+}]_i$ in synaptosomes isolated from rats after postnatal *in vivo* exposure to Pb^{2+} (Sandhir and Gill, 1994).

In this study, we addressed the following unanswered questions. First, what is the effect of Pb^{2+} exposure during development on the presynaptic Ca^{2+} signals produced by impulse activity? Second, if there are changes in presynaptic $[\text{Ca}^{2+}]_i$ regulation, are they correlated with changes in synaptic plasticity? To answer these questions we used the *Drosophila* larval neuromuscular junction (NMJ) one of the preferred systems for studies of the molecular basis for synaptic function, development and plasticity (Keshishian et al., 1996). In addition to its advantages for genetic analyses, the larval motor terminals are identifiable and accessible for electrophysiological and optical studies. Previously, Pb^{2+} exposure was shown to affect the development of behavior in *Drosophila* (Hirsch et al., 2003; Hirsch et al., 2009) and synaptic development at the larval NMJ (Morley et al., 2003).

Materials and Methods

To produce larvae, we added 20 female and male adult flies (wild type, Canton S) to 25 mm vials containing Instant *Drosophila* Medium Formula 4–24 (Carolina Biological Supply Company, Burlington, NC) mixed with either dH_2O (nominal 0 Pb^{2+}), 100 μM Pb acetate in dH_2O or 250 μM Pb acetate in dH_2O . Females thus laid eggs in control or leaded medium;

once eggs hatched the larvae were raised in the same medium until the end of the third larval instar. At this developmental stage larvae leave the medium. We collected only female wandering 3rd instar larvae, identified by their gonads, for Ca^{2+} measurements and synaptic physiology. The doses used were based on previous work showing that 100 μM Pb acetate in dH_2O affected development of the larval motor terminals (Morley et al., 2003), and 250 μM Pb acetate in dH_2O influenced adult fly locomotion (Hirsch et al., 2009).

Loading the Ca^{2+} indicator

To measure $[\text{Ca}^{2+}]_i$, we used the dual-wavelength Ca^{2+} indicator fura-2 (K_d 594 nM) or the single-wavelength indicator, Oregon Green 488 BAPTA-1 (OGB-1; K_d 1180 nM). In both cases, they were coupled to 10,000 MW dextran (Invitrogen, Carlsbad, CA). As in our previous studies (He et al., 2009; Lnenicka et al., 2006a), we loaded the indicator into the motor terminals using the technique developed by Macleod et al., 2002. Briefly, larvae were dissected in Schneider's media (Sigma, St. Louis, MO) and the nerve supplying the third segment was cut. The cut end of the nerve was immediately sucked up into a snug-fitting pipette and then a small volume of indicator (5 mM) was introduced into the end of the pipette tip using very thin tubing. After 40 min, the nerve was removed from the pipette and the preparation was left at room temperature for another hour. The Schneider's solution was then replaced with HL3 saline (Stewart et al., 1994) containing 1 mM Ca^{2+} for physiological measurements. For some experiments, the membrane-permeant, heavy-metal chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN; Invitrogen) was dissolved in ethanol (200 mM) and added to the saline for a final concentration of 100 μM .

Measurement of $[\text{Ca}^{2+}]_i$ changes

The motor terminals were imaged using an upright, fixed-stage BH2 microscope (Olympus) equipped with epifluorescence, differential-interference-contrast optics, a water-immersion 40x Zeiss lens (NA 0.75) and a digital cooled-CCD camera (CoolSNAP HQ, Photometrics, Tucson, AZ). Excitation illumination from a 75W xenon arc lamp was passed through a Lambda-10 Optical Filter Changer (Sutter Instrument Co., Novato, CA). Fura-2 was excited using 360 ± 5 and 380 ± 5 nm bandpass filters (Chroma Technology Corp., Brattleboro, VT); excitation and emission wavelengths were separated with a 410 nm dichroic mirror and the emitted light was passed through a barrier filter of 510 ± 10 nm. For OGB-1, we used a 480 ± 15 nm excitation filter, a 500 nm dichroic mirror and a high-pass 515 nm barrier filter. Images were captured using 100 msec exposures for fura-2. For OGB-1, images were streamed using 20 msec exposures (image size- 50×350 pixels) for single APs and 50 msec (image size- 100×350 pixels) exposures for AP trains. Metafluor 6.1 software (Molecular Devices, Downingtown, PA) was used for image acquisition and to measure fluorescent intensity at synaptic boutons and background fluorescence from the muscle. Metamorph 6.1 software (Molecular Devices) was used to measure bouton widths.

As in a previous study, we estimated the [OGB-1] in the synaptic terminals by measuring the fluorescence intensity for a range of OGB-1 concentrations in capillary tubes with an inside diameter of approximately 20 μm (He et al., 2009). The fluorescent values were normalized to a 20 μm diameter giving a linear plot of [OGB-1] versus fluorescent intensity. For each terminal, the average resting fluorescence was measured at a region of uniform terminal width (apparent diameter) and the fluorescence intensity was normalized to a 20 μm diameter. These capillary tubes contained 40 nM Ca^{2+} ; we assumed a resting $[\text{Ca}^{2+}]_i$ of 40 nM in the motor terminals (Klose et al., 2008). This fluorescent value was then converted to [OGB-1] using the above plot. Capillary tubes with a 20 μm inside diameter were produced from 50 μm capillary tubes (VitroCom, Mountain Lakes, NJ) using a Narishige PN-30 electrode puller (Narishige International USA Inc., East Meadow, NY).

Synaptic physiology

The nerve was stimulated with a suction electrode connected to a S11 stimulator (Grass-Telefactor, West Warwick, RI) and excitatory postsynaptic potentials (EPSPs) were recorded from muscle fibers with sharp electrodes using an Axoclamp 2A amplifier (Axon Instruments Inc., Foster City, CA). pCLAMP 9.2 software and a Digidata 1200A digitizer (Axon Instruments Inc., Foster City, CA) were used to acquire and measure EPSPs.

Data analysis

Sigmaplot 10.0 and SPSS 16.0 (SPSS Inc. Plover, WI) were used for data transformation and statistical analysis. The background fluorescence was subtracted from the bouton fluorescence for each image. For fura-2, we calculated the ratio of fluorescence produced by 360 and 380 nm excitation (360/380). For OGB-1, the percentage change in fluorescence ($\Delta F/F$) was calculated by $100 \times (\text{fluorescence} - \text{initial fluorescence}) / \text{initial fluorescence}$. Single exponentials were fit to the $\Delta F/F$ decay to determine the $[\text{Ca}^{2+}]_i$ decay time constant (τ). We only report $[\text{Ca}^{2+}]_i$ decay τ where the fit to a single exponential gave $r^2 > 0.9$ in order to eliminate noisy measurements. The increase in $[\text{Ca}^{2+}]_i$ during trains of impulses was measured after the $[\text{Ca}^{2+}]_i$ increase reached a plateau by averaging the last 20 measurements of the train. Statistical analyses were performed using t-tests with a Welch correction for unequal variances or an ANOVA followed by a post-hoc analysis (Winer B.J., 1962); p values were adjusted using either a Bonferroni correction when variances were not significantly different or a Games-Howell correction if variances were significantly different (Kirk R.E., 1995). All n values were reported as (# boutons, # animals) unless otherwise noted.

Results

To examine the developmental effect of prolonged Pb^{2+} exposure on intracellular $[\text{Ca}^{2+}]_i$ regulation at synaptic terminals, *Drosophila* larvae were raised from egg stages to the end of the third-instar larval stage in either control media (nominal 0 Pb^{2+} concentration) or in leaded media containing 100 μM or 250 μM Pb^{2+} . In a previous study, the Pb^{2+} burdens were measured in larvae raised in 100 μM Pb^{2+} using inductively-coupled mass spectrometry; this gave values of 12.37 ng Pb^{2+} per larva, which had an average wet weight of 1.6 mg (Morley et al., 2003). The larval mid and hindgut contain about 73% of the Pb^{2+} (Wilson, 2004) and the hemolymph is approximately 40% of wet body weight for insect larvae (Nation, 2001). Thus, the Pb^{2+} concentration in the hemolymph would be 25 μM if all of the Pb^{2+} were in the hemolymph. However, the Pb^{2+} concentration is presumably much less than this since Pb^{2+} would also be distributed in the intracellular fluids and bound to cellular components. If fact, the hemolymph makes up only half of the total larval water content since about 80% of wet larval weight is due to water (Folk 2001). In conclusion, it appears likely that the levels of total Pb^{2+} in the larval hemolymph are micromolar, which falls within the range of Pb^{2+} blood levels observed to influence nervous system development and synaptic plasticity in other organisms (Cline et al., 1996; Gilbert et al., 1996; Lorton and Anderson, 1986; Petit and LeBoutillier, 1979).

In wandering third-instar larvae, we compared $[\text{Ca}^{2+}]_i$ in the Ib motor terminals innervating muscle fibers 6 and 7 (MF 6/7). MF 6/7 are innervated by Is and Ib motor terminals supplied by two separate motor neurons (Hoang and Chiba, 2001; Lnenicka and Keshishian, 2000). The terminals can be distinguished by their different morphology: Ib terminals have large synaptic boutons and Is terminals have small boutons (Kurdyak et al., 1994).

Increase in $[\text{Ca}^{2+}]_i$ produced by impulse activity

The increase in $[\text{Ca}^{2+}]_i$ produced by single APs and trains of APs was examined in control and Pb^{2+} -reared larvae. We used the single-wavelength Ca^{2+} indicator OGB-1, which allowed us to measure the rapid, “volume-averaged” changes in $[\text{Ca}^{2+}]_i$ produced by single APs (Lnenicka

et al., 2006a). The change in OGB-1 fluorescence ($\Delta F/F$) produced by single APs ($\Delta F/F_{AP}$) and AP trains ($\Delta F/F_{train}$) was determined for individual synaptic boutons (Fig 1). In general, the Pb^{2+} -exposed synaptic boutons showed similar $\Delta F/F_{AP}$ amplitudes but larger $\Delta F/F_{train}$ amplitudes than boutons from larvae grown in 0 Pb^{2+} .

The differences in Ca^{2+} transients for control and Pb^{2+} -exposed larvae were quantified by comparing a number of animals. In these experiments, it was necessary to use a similar [OGB-1] in the terminals of control and experimental larvae; the Ca^{2+} indicator acts as a buffer and high concentrations will decrease the amplitude of the $\Delta F/F_{AP}$ and reduce the rate of $[Ca^{2+}]_i$ decay at the end of the Ca^{2+} transient (Neher, 1995). In two experiments, the [OGB-1] was very high ($> 100 \mu M$) and these experiments were not included. For the remaining experiments, the [OGB-1] was similar for terminals from control ($43.3 \pm 7.3 \mu M$, 11 terminals), $100 \mu M$ Pb^{2+} -exposed ($43.3 \pm 8.0 \mu M$, 7 terminals) and $250 \mu M$ Pb^{2+} -exposed larvae ($49.2 \pm 4.1 \mu M$, 7 terminals). In addition, the Ca^{2+} transients can be influenced by bouton size so it was necessary to compare bouton populations that were of similar size (Lnenicka et al., 2006a); bouton width was similar for control ($2.5 \pm 0.1 \mu m$; 94, 11), $100 \mu M$ Pb^{2+} ($2.6 \pm 0.1 \mu m$; 59, 7) and $250 \mu M$ Pb^{2+} terminals ($2.7 \pm 0.1 \mu m$; 121, 7).

The $\Delta F/F_{AP}$ amplitude was not significantly different for Pb^{2+} -reared larvae compared to controls; however, Pb^{2+} -reared larvae showed a significantly larger $\Delta F/F_{train}$ amplitude than controls (Fig. 2). The $\Delta F/F_{train}$ amplitude increased with increasing concentrations of Pb^{2+} . For 20 Hz stimulation, the $\Delta F/F_{train}$ amplitude was 16% and 29% larger than controls for $100 \mu M$ and $250 \mu M$ Pb^{2+} , respectively. An increase in the $[Ca^{2+}]_i$ plateau during an AP train can be produced by greater Ca^{2+} influx or a reduction in the rate of Ca^{2+} extrusion (Tank et al., 1995). Since the $\Delta F/F_{AP}$ amplitudes were similar for control and Pb^{2+} -exposed terminals, it is unlikely that the greater $\Delta F/F_{train}$ amplitude resulted from differences in Ca^{2+} influx. Rather, this difference in amplitude likely reflected differences in Ca^{2+} extrusion.

The decay of $[Ca^{2+}]_i$ after an AP train reflects the rate of Ca^{2+} extrusion: a higher extrusion rate will result in a smaller $[Ca^{2+}]_i$ decay time constant (τ_{decay}). We compared the $[Ca^{2+}]_i$ τ_{decay} for control and Pb^{2+} exposed terminals after single APs and AP trains. There was no clear effect of Pb^{2+} exposure on the $\Delta F/F_{AP}$ τ_{decay} since the τ_{decay} was significantly greater for larvae exposed to $100 \mu M$ Pb^{2+} , but not for those exposed to $250 \mu M$ Pb^{2+} (Fig. 3). For AP trains, Pb^{2+} exposure produced an increase in the τ_{decay} in a dose-dependent manner (Fig. 3, 4). For 20 Hz stimulation, the $\Delta F/F_{train}$ τ_{decay} was 27% and 69% greater than controls for animals exposed to $100 \mu M$ and $250 \mu M$ Pb^{2+} , respectively.

It was possible that the Pb^{2+} -exposed terminals still contained free Pb^{2+} that bound to OGB-1 and compromised our $[Ca^{2+}]_i$ measurements. To test for this, we added $100 \mu M$ TPEN, a membrane-permeant, heavy-metal chelator; TPEN has a much higher affinity for Pb^{2+} than Ca^{2+} and reduces intracellular $[Pb^{2+}]_i$, but not resting $[Ca^{2+}]_i$ (Arslan et al., 1985). In 8 larvae raised in $250 \mu M$ Pb^{2+} , we measured resting OGB-1 fluorescence and $\Delta F/F$ in the same boutons before and after applying TPEN. We found that resting fluorescence ($25.1 \pm 1.4 AU$) and $\Delta F/F_{AP}$ ($8.2 \pm 0.5 \%$) did not significantly change after TPEN addition ($26.4 \pm 1.5 AU$ and $8.2 \pm 0.6 \%$, respectively; $p > 0.10$). Note that the $\Delta F/F_{AP}$ amplitude was small because we included terminals containing high concentrations of OGB-1. This confirms that OGB-1 can be used to accurately measure $[Ca^{2+}]_i$ changes in Pb^{2+} -exposed terminals.

Comparison of resting $[Ca^{2+}]_i$

Pb^{2+} exposure has been shown to produce both an increase and decrease in resting $[Ca^{2+}]_i$ in neurons (Ferguson et al., 2000; Sandhir and Gill, 1994). We performed ratiometric Ca^{2+} measurements using fura-2 to compare resting $[Ca^{2+}]_i$ in synaptic terminals from control and Pb^{2+} -exposed larvae. The fura-2 ratio for experimental and control terminals was also

compared in the presence of TPEN in case there was any free Pb^{2+} remaining in the Pb^{2+} -exposed terminals; fura-2 binds Pb^{2+} with a high affinity and this can produce an increase in the fura-2 ratio (Tomsig and Suszkiw, 1990). The change in indo-1 or fura-2 fluorescence produced by an increase in $[\text{Pb}^{2+}]_i$ was shown to be reversed by the addition of TPEN (Kerper and Hinkle, 1997; Mazzolini et al., 2001).

We found no significant difference in the resting fura-2 ratios for 0 Pb^{2+} (0.35 ± 0.01) and 250 μM Pb^{2+} terminals (0.33 ± 0.02) before adding TPEN ($p > 0.10$). After adding TPEN, there was also no significant difference in the fura-2 ratios for 0 Pb^{2+} (0.36 ± 0.01) and 250 μM Pb^{2+} terminals (0.32 ± 0.02 ; $p > 0.10$). Also, the fura-2 ratio in the Pb^{2+} -exposed terminals was not significantly reduced after adding TPEN ($p > 0.10$). The data were from 4 control and 4 Pb^{2+} -exposed animals and an ANOVA was used for statistical comparisons.

Synaptic facilitation

Synaptic facilitation is dependent on the “residual” Ca^{2+} that equilibrates in the terminal after entering through voltage-dependent Ca^{2+} channels (Zucker and Regehr 2002). Since we have found that an AP train results in a greater buildup of residual Ca^{2+} in Pb^{2+} -exposed terminals, these terminals might show greater synaptic facilitation compared to control terminals. To test this, we measured EPSP amplitude during low-frequency stimulation and during trains of APs delivered at higher frequencies. By performing these experiments on muscle fiber 5, which is innervated by a single axon, we could measure facilitation at a single synapse (Lnenicka et al., 2006b).

We compared synaptic physiology in control larvae and those raised in 250 μM Pb^{2+} since this concentration produced a greater effect on $[\text{Ca}^{2+}]_i$ regulation than 100 μM Pb^{2+} . To determine the EPSP amplitude for single APs, the axon was stimulated at 0.5 Hz, a stimulation frequency that did not produce facilitation. To compare synaptic facilitation, we stimulated the axon at 20 Hz; we previously showed that Pb^{2+} -exposed and control terminals showed clear differences in the buildup of residual Ca^{2+} at this frequency. We found that there was greater synaptic facilitation in Pb^{2+} -exposed animals during 20 Hz stimulation compared to control animals (Fig. 5). We found that the amplitude of the EPSP produced by single APs was not significantly different in 0 Pb^{2+} and 250 μM Pb^{2+} larvae (Fig. 6). However, the EPSP amplitude during 20 Hz stimulation was significantly greater for Pb^{2+} -exposed larvae compared to controls.

The differences in the EPSP amplitude seen during 20 Hz stimulation appear to be mainly due to differences in the buildup of synaptic facilitation. During a train of APs, the enhancement of EPSP amplitude can result from the buildup of facilitation plus the onset of the longer-lasting augmentation and post-tetanic potentiation (PTP). Synaptic facilitation has a decay time constant of 10s to 100s of milliseconds, augmentation decays with a time constant of 5–10 sec. and PTP has a decay time constant of 30 sec to several minutes (Magleby and Zengel, 1976; Magleby and Zengel, 1975; Mallart and Martin, 1967). The differences in EPSP amplitude for Pb^{2+} -exposed and control larvae were nearly gone 2 seconds after the end of the 20 Hz train (Fig. 5). Thus, the differences between Pb^{2+} -exposed and control terminals are only seen during synaptic facilitation and not during the augmentation and PTP phases. Overall, the synaptic facilitation during the 20 Hz train was significantly greater in Pb^{2+} -exposed larvae than in control larvae (Fig. 6).

Discussion

Pb^{2+} exposure influences the $[\text{Ca}^{2+}]_i$ increase produced by APs

We found that the Ca^{2+} transient produced by single APs was not influenced by chronic Pb^{2+} exposure; however, Pb^{2+} exposure resulted in a larger Ca^{2+} transient during AP trains.

The $\Delta F/F_{AP}$ amplitude was similar in control synaptic boutons and those exposed to 100 or 250 μM Pb^{2+} . Since the amplitude of the single-AP Ca^{2+} transient is determined by the amount of Ca^{2+} influx and the concentration of fast Ca^{2+} buffers, it appears that Ca^{2+} influx was similar in control and Pb^{2+} -exposed terminals. Alternatively, there could have been Pb^{2+} -related changes in Ca^{2+} influx that were counterbalanced by changes in fast Ca^{2+} buffering. This seems unlikely since changes in Ca^{2+} buffering would result in a change in the $\Delta F/F_{AP} \tau_{decay}$ (Neher, 1995) and we did not see a consistent effect of Pb^{2+} exposure on the $\Delta F/F_{AP} \tau_{decay}$. The inhibition of Ca^{2+} influx by acute Pb^{2+} exposure (nanomolar to micromolar) has been demonstrated for a variety of voltage-dependent Ca^{2+} channels in both invertebrate (Audesirk and Audesirk, 1989; Busselberg et al., 1990) and mammalian voltage-dependent Ca^{2+} channels (Audesirk and Audesirk, 1991; Audesirk and Audesirk, 1993; Busselberg et al., 1993; Evans et al., 1991; Peng et al., 2002). In most cases, the inhibition of Ca^{2+} influx by Pb^{2+} was reversible (Audesirk, 1993) and we assume that any inhibitory effects of Pb^{2+} were washed out before we performed our measurements. Thus, our measurements do not rule out the possibility that Pb^{2+} in the hemolymph inhibited Ca^{2+} influx *in vivo*.

For AP trains, chronic Pb^{2+} exposure resulted in a linear dose-dependent increase in the $\Delta F/F_{train}$ amplitude and τ_{decay} . We assume that both of these effects resulted from a decrease in the rate of Ca^{2+} extrusion by the PMCA. During an AP train, the buildup of $[Ca^{2+}]_i$ results in greater Ca^{2+} extrusion until Ca^{2+} influx and efflux per unit time are equal and the plateau is reached (Tank et al., 1995); a reduction in the Ca^{2+} extrusion rate constant will result in a greater $\Delta F/F_{train}$ amplitude. At the end of the train, the $[Ca^{2+}]_i$ decay reflects the rate of Ca^{2+} extrusion and a lower extrusion rate will result in a greater $\Delta F/F_{train} \tau_{decay}$. The PMCA is responsible for Ca^{2+} extrusion from these larval synaptic terminals; inhibition of the PMCA resulted in an increase in $\Delta F/F_{train}$ amplitude and the $\Delta F/F_{train} \tau_{decay}$ and immunostaining showed localization of the PMCA at the NMJ (Lnenicka et al., 2006a). It appears unlikely that Pb^{2+} also acts on other Ca^{2+} -clearance mechanisms to produce the increase in $\Delta F/F_{train}$ amplitude since blocking Ca^{2+} extrusion by the Na/Ca exchanger or Ca^{2+} uptake by mitochondria or ER did not result in an increase in $\Delta F/F_{train}$ amplitude in a previous study (Lnenicka et al., 2006a).

There was no clear and consistent effect of Pb^{2+} exposure on the $\Delta F/F_{AP} \tau_{decay}$ (the $\Delta F/F_{AP} \tau_{decay}$ increased significantly for the 100 μM Pb^{2+} group and then dropped for the μM Pb^{2+} group). This may be because the PMCA plays a greater role in determining the $\Delta F/F_{train} \tau_{decay}$ than the $\Delta F/F_{AP} \tau_{decay}$. This is supported by the finding that blocking the PMCA had a greater effect on $\Delta F/F_{train} \tau_{decay}$ than on $\Delta F/F_{AP} \tau_{decay}$ (Lnenicka et al., 2006a). Factors other than the PMCA may play a role in the $\Delta F/F_{AP} \tau_{decay}$; e.g., studies of crayfish motor terminals indicate that slow Ca^{2+} buffers play a role in the $\Delta F/F_{AP} \tau_{decay}$ (Lin et al., 2005).

Pb^{2+} exposure has been shown to influence PMCA activity in other systems. Acute exposure to micromolar concentrations of Pb^{2+} reduced the activity of the PMCA in humans and rats (Bettaiya et al., 1996; Mas-Oliva, 1989; Sandhir and Gill, 1994). The *in vitro* studies indicate that Pb^{2+} inhibits PMCA activity by binding directly to the PMCA (Mas-Oliva, 1989; Sandhir and Gill, 1994). Studies examining the effect of *in vivo* chronic Pb^{2+} application have shown a persistent inhibition of PMCA activity; i.e., PMCA activity was inhibited when measured in nominal Pb^{2+} -free solutions. For example, the PMCA activity in erythrocytes from mothers and newborns was negatively correlated with hair Pb^{2+} levels (Campagna et al., 2000). In addition, rats exposed to Pb^{2+} intragastrically (50 mg/kg body weight) for 8 weeks showed a 31% decrease in PMCA activity per total protein for synaptic plasma membranes isolated from the brain (Sandhir and Gill, 1994). Our results also suggest a persistent effect of Pb^{2+} on PMCA activity and our experiments using TPEN showed no evidence for free Pb^{2+} remaining in the synaptic terminals. The persistent effects of chronic Pb^{2+} exposure could be due to irreversible binding of Pb^{2+} to the PMCA. Alternatively, Pb^{2+} exposure could have reduced the expression

of PMCA. The level of $[Ca^{2+}]_i$ is one factor regulating PMCA expression (Guerini et al., 1999) and it may be that Pb^{2+} exposure decreased PMCA expression by reducing Ca^{2+} influx through Ca^{2+} channels.

In mammals, there are 4 PMCA genes and over 20 possible splice variants (Strehler and Treiman, 2004; Strehler and Zacharias, 2001) expressed in a tissue- and development-specific manner; all cell types express at least one of the isoforms (Strehler and Treiman, 2004). Nervous tissue appears to show the greatest abundance and variety of PMCA expression: many of the isoforms have been demonstrated in the brain (Strehler and Treiman, 2004). *Drosophila* has fewer PMCA genes and isoforms than found in mammals. Based upon the annotation of the genome, and BLASTp analysis of a mouse PMCA (PMCA-4) against the *Drosophila* annotated protein sequences, *Drosophila* has only a single highly homologous PMCA gene (CG42314) and 6 potential isoforms (E value = 0).

We found no effect of chronic Pb^{2+} exposure on resting $[Ca^{2+}]_i$ and this appears unexpected since the PMCA plays an important role in maintaining resting $[Ca^{2+}]_i$. However, previous *in vitro* studies have reported an increase in resting $[Ca^{2+}]_i$ during exposure to 5 μM Pb^{2+} (Schanne et al., 1989) and a decrease in resting $[Ca^{2+}]_i$ (Ferguson et al., 2000) during exposure to 0.1 μM Pb^{2+} . This apparently occurs because Pb^{2+} can stimulate PMCA activity as well as inhibit it. Normally, an increase in $[Ca^{2+}]_i$ activates the PMCA through the formation of Ca-calmodulin, which binds to the C-terminal tail of the PMCA releasing it from autoinhibition (Strehler and Treiman 2004). Pb^{2+} can substitute for Ca^{2+} in calmodulin activation (Goldstein and Ar, 1983; Habermann et al., 1983; Kern et al., 2000) and this can result in an increase in PMCA activity (Ferguson et al., 2000). Synaptosomes isolated from rats exposed to Pb^{2+} *in vivo* (50 mg/kg body weight) showed an increase in resting $[Ca^{2+}]_i$ due to reduced PMCA activity even though there was also an increase calmodulin activity (Sandir and Gill, 1994). The balance of PMCA inhibition and activation likely determines the effect of Pb^{2+} on resting $[Ca^{2+}]_i$; this likely depends on the Pb^{2+} concentration since activation of calmodulin occurs at nanomolar concentrations and PMCA inhibition at micromolar concentrations (Ferguson et al., 2000). In our studies, there may have been an equal balance of PMCA inhibition and excitation during resting $[Ca^{2+}]_i$; however, the effects of PMCA inhibition may have predominated at higher $[Ca^{2+}]_i$. For example assuming Michaelis-Menton kinetics and fewer functional PMCA (lower V_{max}) in Pb^{2+} -exposed larvae, the effects of PMCA inhibition on the steady-state $[Ca^{2+}]_i$ would be accentuated at a higher $[Ca^{2+}]_i$. As Ca^{2+} influx increased during AP trains, the steady-state $[Ca^{2+}]_i$ concentration would increase more rapidly in Pb^{2+} -exposed animals since the PMCA would approach saturation at a lower $[Ca^{2+}]_i$.

Synaptic facilitation

We tested whether the change in $[Ca^{2+}]_i$ regulation resulting from Pb^{2+} exposure produced changes in synaptic plasticity. We found that during a train of impulses there was a greater increase in EPSP amplitude for Pb^{2+} -exposed terminals than for control terminals. This activity-dependent synaptic enhancement is the most common form of synaptic plasticity and is found at most, if not all synapses (Zucker and Regehr, 2002). It results from an increase in transmitter release and can be divided into synaptic facilitation, augmentation and PTP based upon differences in time course. It appeared that differences in synaptic enhancement seen for control and Pb^{2+} -exposed terminals were due to differences in the buildup of synaptic facilitation.

The greater synaptic facilitation seen at the Pb^{2+} -exposed terminals is likely due to the greater increase in $[Ca^{2+}]_i$ seen at these terminals. Although the mechanisms underlying synaptic enhancement are not understood, studies of synapses in vertebrates and invertebrates have shown that facilitation, augmentation and PTP appear to be dependent upon residual Ca^{2+} (Zucker and Regehr, 2002). For example, adding the Ca^{2+} chelator ethylene glycol tetraacetic

acid (EGTA) alters the buildup and decay of Ca^{2+} and synaptic enhancement during a train at mammalian central synapses and the lobster NMJ (Ogawa et al., 2000; Regehr et al., 1994).

Previous studies examining the effects of *in vivo* Pb^{2+} exposure on synaptic plasticity have mainly focused on long-term potentiation (LTP). Postnatal Pb^{2+} exposure increased the threshold for producing LTP, decreased its amplitude and reduced the duration of LTP in the rat hippocampus, possibly due to the effect of Pb^{2+} on the NMDA receptor (Gilbert et al., 1996; Gilbert and Mack, 1998; Lasley et al., 1993; Ruan et al., 1998). However, the effect of Pb^{2+} exposure on short-term plasticity may differ since exposure to levels of Pb^{2+} that impaired LTP resulted in an increase in the amplitude of short-term potentiation (STP) in the rat hippocampus (Grover and Frye, 1996). Since STP may involve presynaptic mechanisms (Lauri et al., 2007), the increase in STP could involve an increase in residual Ca^{2+} as seen at the larval NMJ.

The changes that we observe in $[\text{Ca}^{2+}]_i$ regulation and synaptic facilitation at the neuromuscular synapses are likely to occur at synapses in the central nervous system since the PMCA plays an important role in Ca^{2+} extrusion from synaptic terminals in the central nervous (Juhaszova et al., 2000) (Morgans et al., 1998) and synaptic facilitation at central synapses is dependent on residual Ca^{2+} (Atluri and Regehr, 1996). In fact, reduced PMCA activity has been shown to enhance synaptic facilitation at central synapses; these studies involved blocking PMCA activity pharmacologically or knocking out expression of one of the PMCA isoforms (Empson et al., 2007; Jensen et al., 2007).

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Figure 1.

Ca^{2+} transients recorded from synaptic boutons in control and Pb^{2+} -exposed animals. *Left:* Ib terminals on MF 6/7 filled with OGB-1 from control larvae and those exposed to Pb^{2+} . The arrows point to two typical synaptic boutons where Ca^{2+} transients were measured. *Right:* Typical Ca^{2+} transients for single APs and AP trains seen at control and Pb^{2+} -exposed (250 μM) boutons. The $\Delta\text{F}/\text{F}_{\text{AP}}$ was similar for control and Pb^{2+} -exposed synaptic boutons; however, the $\Delta\text{F}/\text{F}_{\text{train}}$ was generally larger in Pb^{2+} -exposed boutons compared to controls. Calibration: Single AP- 20% $\Delta\text{F}/\text{F}$, 0.4 sec; AP train- 20% $\Delta\text{F}/\text{F}$, 2 sec.

Figure 2.

The Ca²⁺ transient amplitude in synaptic boutons from control larvae and those exposed to 100 or 250 μM Pb²⁺. The OGB-1 ΔF/F was measured at the peak of the Ca²⁺ transient for single APs (ΔF/F_{AP peak}) and at the plateau for AP trains (ΔF/F_{train plateau}). *Top*: The ΔF/F_{AP peak} was not significantly different for Pb²⁺-exposed larvae compared to control larvae. *Middle*: The ΔF/F_{train plateau} during 10 Hz stimulation was significantly greater for Pb²⁺-exposed animals compared to control ones. *Bottom*: The ΔF/F_{train plateau} during 20 Hz stimulation was significantly greater for the Pb²⁺-exposed larvae compared to control larvae. Since data for the different stimulation rates are not independent, we used the ANOVA routines of SPSS to analyze amplitude separately for single APs, 10Hz and 20Hz. For a single AP, there was no

significant overall effect of Pb^{2+} -exposure ($F=2.70$; $df=1$; $p>0.05$), but there were significant linear effects of dose for both 10Hz ($F=54.21$; $df=1$; $p<0.001$) and 20Hz ($F=28.12$; $df=1$; $p<0.001$); i.e., the height of the plateau for 10Hz and 20Hz was proportional to the lead dose. Results of a post-hoc analysis showed that for both 10Hz and for 20Hz, the observed plateaus for 100 μM and for 250 μM Pb^{2+} were significantly greater than the plateau for the controls. Post-hoc analysis used Games-Howell correction for multiple comparisons because group variances were significantly different based on the Levene statistic. Significantly greater than control: *** $p<0.001$. n values represent (#boutons, #animals).

Figure 3.

The Ca²⁺ transient decay time constant in synaptic boutons from control and Pb²⁺-exposed animals. The τ_{decay} of the $\Delta F/F$ was measured after a single AP ($\Delta F/F_{\text{AP}} \tau_{\text{decay}}$) or at the end of an AP train ($\Delta F/F_{\text{train}} \tau_{\text{decay}}$). *Top:* The $\Delta F/F_{\text{AP}} \tau_{\text{decay}}$ for larvae raised in 100 μM Pb²⁺, but not 250 μM Pb²⁺, was significantly greater than that for control animals. *Middle:* For 10 Hz stimulation, the $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ increased with increasing Pb²⁺ concentration; the $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ was significantly larger than control for 250 μM Pb²⁺, but not for 100 μM Pb²⁺. *Bottom:* The $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ increased with increasing Pb²⁺ concentration for 20 Hz stimulation; the $\Delta F/F_{\text{train}} \tau_{\text{decay}}$ was significantly greater than control for both 100 and 250 μM Pb²⁺. For single APs, the ANOVA gave no significant linear effect of dose ($F=0.264$; $df=1$;

$p > 0.05$) but there was a significant quadratic effect of dose ($F = 8.23$; $df = 1$; $p < 0.01$); i.e., the $\Delta F/F_{AP} \tau_{decay}$ was not proportional to the lead dose. Results of a post-hoc analysis using Bonferroni correction for multiple comparisons (variances not significantly different) showed that the $\Delta F/F_{AP} \tau_{decay}$ for Pb^{2+} -exposed larvae was significantly greater than for controls for 100 $\mu M Pb^{2+}$ but not for 250 $\mu M Pb^{2+}$. For 10Hz there was a significant linear effect of dose ($F = 11.12$; $df = 1$; $p < 0.002$) as there was for 20 Hz ($F = 57.55$; $df = 1$; $p < 0.001$). There were no significant higher-order effects for these two groups; $\Delta F/F_{train} \tau_{decay}$ was thus proportional to dose. For the 10Hz data, results of a post-hoc analysis using Bonferroni correction for multiple comparisons (variances not significantly different) showed that the $\Delta F/F_{train} \tau_{decay}$ for Pb^{2+} -exposed larvae was significantly greater than for controls for 250 $\mu M Pb^{2+}$ but not for 100 $\mu M Pb^{2+}$. For the 20Hz data, results of a post-hoc analysis using Games-Howell correction for multiple comparisons (variances significantly different) showed that the $\Delta F/F_{train} \tau_{decay}$ for Pb^{2+} -exposed larvae was significantly greater than for controls for both 100 $\mu M Pb^{2+}$ and 250 $\mu M Pb^{2+}$. Significantly greater than control: * $p < .05$, ** $p < .01$, *** $p < .001$. n values represent (#boutons, #animals).

Figure 4.

Ca^{2+} transient decay after 20 Hz AP train normalized to the final response at the end of the AP train. The mean normalized $\Delta\text{F}/\text{F}_{\text{train}}$ decay was compared for animals exposed to 0 $\mu\text{M Pb}^{2+}$ (68, 10), 100 $\mu\text{M Pb}^{2+}$ (53, 7) and 250 $\mu\text{M Pb}^{2+}$ (74, 7). The $\Delta\text{F}/\text{F}_{\text{train}}$ decays more slowly in Pb^{2+} -exposed synaptic boutons in a dose-dependent manner. n values represent (#boutons, #animals).

Figure 5.

EPSPs recorded from muscle fibers in control and Pb²⁺-exposed larvae during 20 Hz stimulation. *Top:* Typical EPSPs recorded from muscle fiber 5 during 20 Hz stimulation. The EPSPs were larger in larvae raised in media containing 250 μM Pb²⁺ compared to EPSPs from larvae raised in 0 Pb²⁺. *Bottom:* Mean EPSP amplitudes recorded from muscle fiber 5 during 20 Hz stimulation. The data are from 24 muscle fibers in 7 larvae raised in control media (0 Pb²⁺) and 29 muscle fibers in 8 larvae grown in media containing 250 μM Pb²⁺. The initial response (0 sec) was the average of 20 EPSPs produced by 0.5 Hz stimulation. The subsequent points are the average of 10 successive EPSPs during 20 Hz stimulation. The final 2 points are individual EPSPs produced by 0.5 Hz stimulation. Twenty Hz stimulation resulted in greater facilitation of EPSP amplitude in Pb²⁺-exposed larvae compared to controls.

Figure 6.

Mean EPSP amplitudes and synaptic facilitation in control larvae and those exposed to 250 μM Pb²⁺. *Left:* The EPSP amplitude produced by single APs (0.5 Hz) was not significantly different in 0 Pb²⁺ and 250 μM Pb²⁺ larvae. During 20 Hz stimulation, the EPSP amplitude was significantly larger for Pb²⁺-exposed larvae compared to control larvae. The mean value for 20 Hz was determined from a train of 200 stimuli and the 0.5 Hz value was the mean of 20 EPSPs. *Right:* Synaptic facilitation was calculated for each experiment and the Pb²⁺-exposed larvae showed significantly greater facilitation than control larvae. Facilitation (%) = (20 Hz EPSP amplitude/0.5 Hz EPSP amplitude) × 100. The mean values were obtained from 29 muscle fibers in 8 Pb²⁺-exposed larvae and 24 muscle fibers in 7 control larvae. * p < 0.05, ** p < 0.01; t-test with Welch correction.